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Glutaric aciduria type I: Pathomechanisms of neurodegeneration

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Summary: In organotypic corticostriatal and hippocampal slice cultures from rat brain, 3-hydroxyglutaric acid but not glutaric and glutaconic acids induced neurodegeneration by activation of NMDA receptors. Electrophysiological investigations (*Xenopus laevis* oocytes expressing glutamate receptors; rat mixed cortex culture) revealed no direct interaction of 3-hydroxyglutaric acid with glutamate receptors. We speculate that 3-hydroxyglutaric acid induces a mild energy deprivation that interferes with the voltage-dependent Mg^{2+} -block of NMDA receptors.

We investigated whether glutaric, 3-hydroxyglutaric (3-OH-glutaric), and/or glutaconic acids produce glutamate receptor-mediated neurotoxicity in corticostriatal, cortical and hippocampal slice cultures from postnatal rat brain.

Glutamate receptors fall principally into two broad categories: the ionotropic receptors that are coupled to ion channels and the metabotropic receptors that effect signal transduction via activity of G-protein-linked enzymatic processes. Within each broad family, numerous receptor subtypes have been identified. The ionotropic receptors are divided into two families, the NMDA receptors interacting with *N*-methyl-D-aspartate and the non-NMDA receptors preferentially activated by either kainate or by AMPA. All ligand-gated ion channels are permeable to sodium. The NMDA receptors are especially permeable to calcium and, in contrast to other glutamate receptors, possess a voltage-dependent Mg^{2+} -block.

Glutamate receptors play a physiological role in excitatory neurotransmission and in brain maturation (synaptogenesis, dendritic growth) but also in pathological mechanisms (Olney 1993; Bittigau and Ikonomidou, 1997).

The concept of excitotoxicity describes the potential cell damage induced by excitatory amino acids like glutamate via NMDA and non-NMDA receptors (Olney 1980). Morphologically, a massive swelling and vacuolization of the cytoplasm of postsynaptic neurons due to sodium and water influx is observed. A secondary increase of intracellular calcium concentrations due to influx and release from intracellular stores with inhibition of ATP-synthase, activation of proteases and lipases and activation of nitric-oxide synthase is thought to lead to cell death (Olney 1993, Bittigau and Ikonomidou 1997). Most recently it was shown (McDonald et al 1998) that the activation of the AMPA-receptors GluR₂/R₃ may also induce degeneration of glial cells.

Excessive activation of glutamate receptors can be mediated by either elevated agonist concentrations or cellular energy depletion even in the presence of normal agonist concentrations. Under resting conditions, the glutamate concentration in the extracellular space is lower by several factors than in the postsynaptic cytoplasm. The gradient is maintained by distinct carriers. With lack of energy, the glutamate carriers may not function properly or may even reverse their operation and release glutamate into the extracellular space, leading to activation of different glutamate receptor subtypes. Neurodegeneration with release of glutamate enhances this mechanism. There is evidence that the acute degenerative changes following status epilepticus, hypoglycinaemia and cerebral ischaemia may partly result from endogenously released excitatory amino acids (Nicholls and Attwell 1990). The *in vitro* work of Henneberry and colleagues (1989) suggested that cellular energy deficits may lead to activation of the NMDA channel by a reduction of the resting membrane potential: this induces a release of the voltage-dependent Mg²⁺-block of the channel and allows ions to enter the cell persistently. This hypothesis is supported by experimental results that show that the mitochondrial toxins MPP (Turski et al 1991), 3-nitropropionic acid (Ludolph et al 1992) and cyanide (Zeevalk and Nicklas 1991, 1992) induce morphological changes comparable to those associated with excitotoxic damage.

Metabolic disorders that are good candidates for excitotoxicity are sulphite-oxidase deficiency and homocystinuria. Olney and colleagues (1971) reported that cysteine S-sulphate induces excitotoxic cell damage and we have recently shown that homocysteic and homocysteine sulphinic acids activate different glutamate receptor subtypes (Flott-Rahmel et al 1998).

Several findings cause us to speculate that excitotoxic mechanisms also may play a role in glutaric aciduria type I. Acute encephalopathic crises, brain/brainstem ganglia oedema and urinary excretion of lactate and dicarboxylic acids indicate mitochondrial damage or energy crisis (Gregersen and Brandt 1979). Neuronal degeneration is found in brain areas with high glutamate receptor density, and postsynaptic vacuolization of neurons has been described (Goodman et al 1977; Soffer et al 1992). Glutaric and hydroxyglutaric acid inhibit the activity of glutamate decarboxylase with a K_i of 1–3 mmol/L, the range of cerebral glutaric acid concentrations found in brain tissue of patients (Stokke et al 1976; Taberner et al 1977). Reduced activity of glutamate decarboxylase as well as reduced GABA concentrations were found in one patient, but surprisingly only in brainstem ganglia, using

control values from the literature (Leibel et al 1980). At higher concentrations ($K_i \sim 10$ mmol/L), glutaric acid inhibits the uptake of glutamate into synaptosomes (Bennett et al 1973). In addition, glutaric acid has a similar structure to glutamate (Watkins 1991).

MATERIAL AND METHODS

Organotypic slice cultures: Hippocampus, motor cortex or striatum with overlying cortex slices were prepared of 6-day-old Wistar rats (Baker et al., 1992; Gähwiler, 1981). The tissue was cut into 350 μ m thick slices with a McIlwain tissue chopper and incubated in Minimum Essential Medium (MEM) for 30 min at 4°C.

The slices were cultivated on membranes as described by Stoppini and colleagues (1991) using either membranes from Nunc (0.2 μ m) or from Millipore (0.4 μ m) that inserted into 6-well culture dishes. Although both membrane types were suitable for culturing the slices, Nunc membranes produced no background fluorescence during propidium iodide staining. Millipore membranes, however, were easier to handle for sectioning. The culture medium consisted of 50% MEM/Hanks' balanced salt solution (Frotscher and Heimrich 1993) that does not contain additional glucose. This medium was found to give better culture results compared with other media tested (Gähwiler 1984; Vornov et al 1991) as examined by propidium iodide staining. LDH measurement, described as an alternative for estimation of cell damage (Koh and Choi 1987), was not found to be sensitive enough to reflect cell damage in our culture system.

Administration of glutamate receptor agonist or metabolites: Short-term incubation experiments with glutamate receptor agonists were performed in Hanks' balanced salt solution (Vornov et al 1991) containing either glutamate (100 μ mol/L), or kainate (100 μ mol/L), or NMDA plus glycine (100 μ mol/L + 10 μ mol/L) using slices that had been cultivated for 7 days. After 30 min incubation with agonists, cells were transferred to serum-free medium (75% MEM, 25% BME, and 2 mmol/L glutamine) for a 24 h recovery period. Incubations in the presence of glutaric acid (Sigma), 3-OH-glutaric acid, and glutaconic acid (Fluka) (1–5 mmol/L each) were performed in either HBSS or in HBSS without glucose and Mg^{2+} for 4 h followed by 24 h of recovery. 3-OH-Glutaric acid was synthesized from its dimethyl ester (Aldrich) by alkaline hydrolysis (purity > 97%, GC-MS).

In protection experiments, cultures were pretreated with the noncompetitive NMDA receptor antagonist MK-801 (20 μ mol/L) and the non-NMDA receptor antagonist CNQX (10 μ mol/L) for 20 min prior to co-incubation with the respective agonists/metabolites. Long-term experiments were started by adding the metabolites directly to the culture medium at day 1 or 3 of cultivation (for 7 days) or day 7 of cultivation. The medium was changed every second day.

Nuclei of degenerating cells were visualized by propidium iodide (Sigma) staining (Vornov et al 1991).

Examination of energy levels: The effects of glutaric acid, 3-OH-glutaric acid and glutaconic acid (1–5 mmol/L) on ATP levels were tested in the absence or presence of 3-nitropropionic acid (10 μ mol/L–1 mmol/L). Incubation times were 4 h and 24 h.

ATP and creatine phosphate levels were examined using a bioluminescence assay (Lundin et al 1976).

The activity of the respiratory chain enzymes was measured as described by Das (1998).

Glutaryl-CoA dehydrogenase activity in cultures and tissue concentrations of glutaric acid: Glutaryl-CoA dehydrogenase activity was examined in organotypic striatal, cortical and hippocampal cultures as well as in uncultured brain tissue by measurement of CO₂ release from [1,5-¹⁴C]glutaryl-CoA (Christensen 1983). Enzyme activity in organotypic cultures was relatively high compared with tissue that had not been in culture. Enzyme activities (μ mol/h per g protein) were as follows: (a) in cultured tissue, striatal 8.95 (SD 0.78), cortical 8.75 (SD 0.21), hippocampal 10.1 (SD 0.61); (b) in uncultured tissue, striatum 3.85 (SD 0.39), cortex 2.2 (SD 0.62), hippocampus 3.58 (SD 1.16), $n = 3$ each.

Glutaric acid concentrations were measured using stable-isotope dilution gas chromatography–mass spectroscopy (Jakobs 1989). Although glutaryl-CoA dehydrogenase is expressed in organotypic slice cultures, incubation with glutaric acid (1 mmol/L, 4 h or 10 days) resulted in tissue concentrations of glutaric acid up to 300 μ g/g wet weight, which is higher than that measured in brain autopsy material derived from GA I patients (88–165 μ g/g; Goodman and Frerman 1995). These findings indicate, that although glutaryl-CoA dehydrogenase activity is expressed, incubation with glutaric acid leads to elevated metabolite concentrations within cell cultures.

Histological observations: For histological analysis, slice cultures on Millipore membranes were fixed in Karnovsky's solution (2.2% glutaraldehyde and 2.5% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.35). The tissue/membrane was postfixed in OsO₄, dehydrated in alcohol, and embedded in Epon. Semithin horizontal sections were cut starting from the surface of the slices. Sections were stained with toluidine blue for morphological orientation and examined by light microscopy.

Electrophysiological investigations: To investigate whether glutaric acid, 3-OH-glutaric acid, and glutaconic acid exhibit any influence on glutamate receptors, oocytes of *Xenopus laevis* were injected with mRNA isolated from neocortical rat brain (day 21, including frontal brain; Musshoff et al 1994). Expression of glutamate receptors was tested by electrophysiological measurements in buffer supplemented with either NMDA plus glycine (100 μ mol/L + 10 μ mol/L) or AMPA (100 μ mol/L) or kainate (100 μ mol/L). The effects of glutaric, 3-OH-glutaric and glutaconic acid were tested by adding these substances (100 μ mol/L, 1 mmol/L, 3 mmol/L) to the medium in the absence or presence of the respective glutamate receptor agonists. Membrane cur-

rents were measured using the conventional two-electrode voltage clamp technique. The holding potential was -70 mV.

RESULTS

A 30 min incubation of cortical/corticostriatal cultures with glutamate (1 mmol/L), kainate (100 μ mol/L) or NMDA (100 μ mol/L) followed by a period of 24 h of recovery resulted in massive cell damage. The same results were obtained in hippocampal cultures incubated with glutamate (1 mmol/L), kainate (100 μ mol/L) and NMDA (100 μ mol/L). After 30 min incubation (24 h recovery), similar lesion patterns to those described for hippocampal roller cultures (Tasker et al 1992; Vornov et al 1991) could be observed. Cell death could be attenuated by co-incubation of the cultures with MK 801 (20 μ mol/L) or CNQX (10 μ mol/L), indicating that glutamate receptor activation was involved.

Incubation of cortical/corticostriatal and hippocampal slice cultures with glutaric acid or glutaconic acid (1–5 mmol/L) did not lead to remarkable propidium iodide staining. This was found for both short term (4 h) and long-term incubation (10 days) experiments.

In contrast, incubation of corticostriatal and hippocampal cultures with 3-OH-glutaric acid for more than 4 h exhibited a dose-dependent neurotoxic effect as assessed by propidium iodide staining. Toxicity in hippocampal cultures appeared at concentrations of 1.5 mmol/L 3-OH-glutaric acid (Figure 1b). With increasing concentration (3 mmol/L), the effect became more prominent (Figure 1c) and was accompanied by total disintegration of the slice culture (5 mmol/L). Propidium iodide label was completely attenuated by co-incubation with MK 801, indicating that cell death is mediated by the activation of NMDA receptors. Incubation of corticostriatal cultures with 3-OH-glutaric acid again led to a dose-dependent neurodegeneration, starting within the striatum at medium concentrations of 1.5 mmol/L. Morphologically, 3-OH-glutaric acid induced massive swelling and loss of neurons (Figure 2) (Flott-Rahmel et al 1997).

With simple immunostaining (glutamate decarboxylase), no specific degeneration of GABAergic neurons was found.

At high concentrations (5 mmol/L) and incubation for more than 24 h, glutaric, 3-OH-glutaric and glutaconic acids produced cell swelling within the glia layer which could not be prevented by co-incubation with NMDA receptor and non-NMDA receptor antagonists (MK 801, CNQX) (Figure 3).

Administration of 3-OH-glutaric and glutaconic acid led to a 10–30% reduction of ATP levels in corticostriatal/hippocampal slice cultures.

Treatment of corticostriatal/hippocampal slices with 1 mmol/L 3-nitropropionic acid, an inhibitor of complex II of the respiratory chain, led to intense propidium iodide staining and lowered ATP levels by about 80% of control values. Cell degeneration could be attenuated by co-incubation with MK 801 (20 μ mol/L). A combination of subtoxic concentrations of 3-nitropropionic acid (10 μ mol/L; \sim 25% reduction of intracellular ATP levels) with 3-OH-glutaric and glutaconic acids (1–

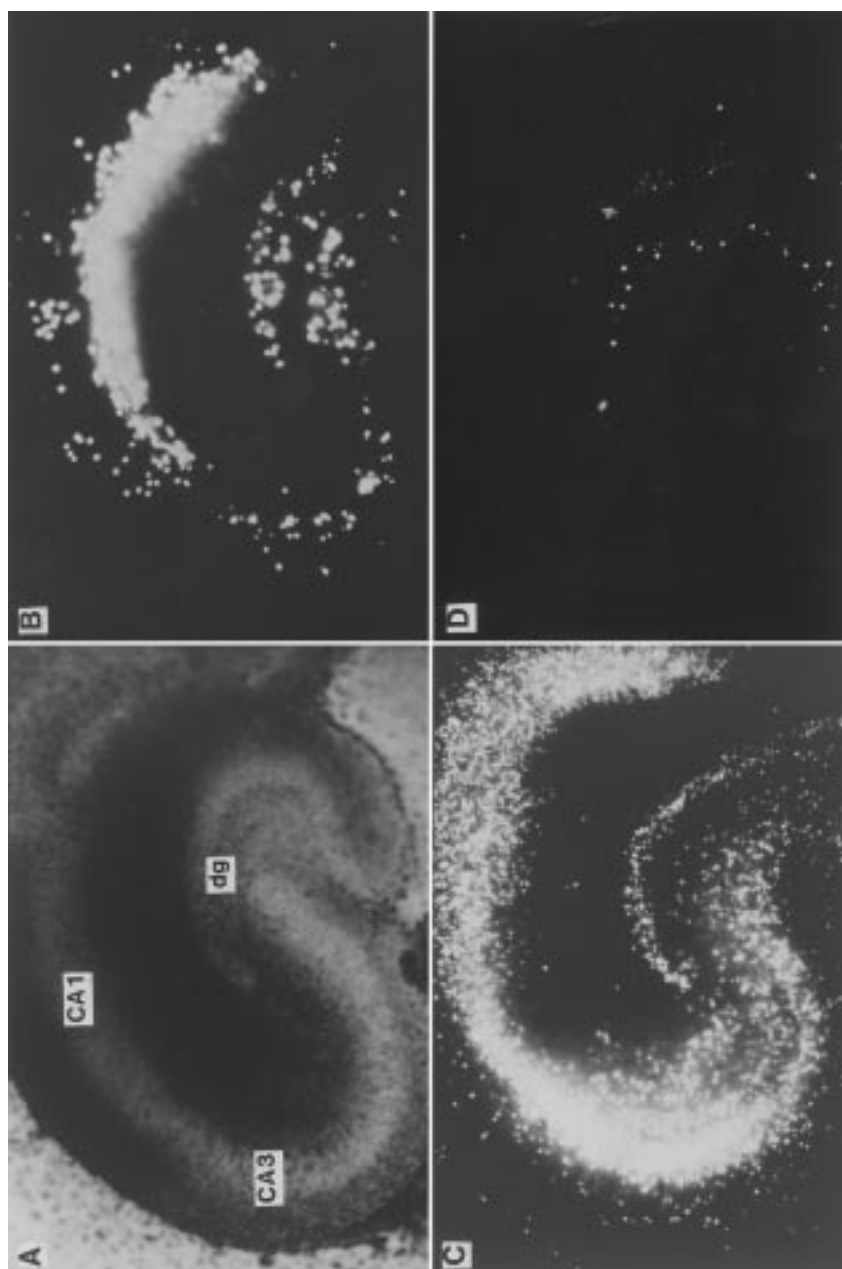


Figure 1 (A) Hippocampal slice culture, CA₁ = CA₁, region of the pyramidal cell layer; CA₃ = CA₃, region of the pyramidal cell layer; dg = dentate gyrus. (B) Hippocampal culture, fluorescence microscopy, 1.5 mmol/L 3-OH-glutaric acid. (C) Same culture as in (B), 3 mmol/L 3-OH-glutaric acid. (D) Same culture as in (B) and (C), 3 mmol/L 3-OH-glutaric acid plus 20 μmol/L MK-801

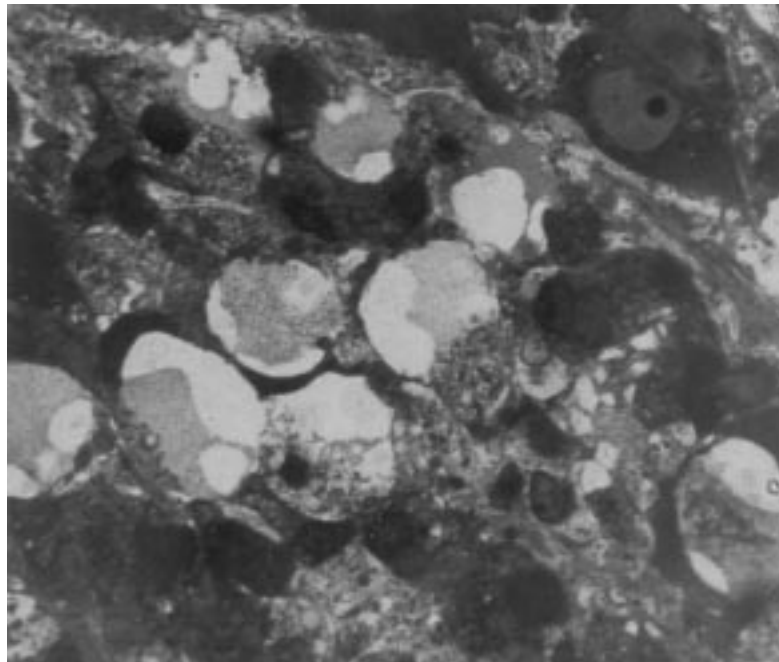


Figure 2 Hippocampal culture, vacuolation of neurons following incubation with 3 mmol/L 3-OH-glutaric acid. Toluidine blue staining $\times 500$

5 mmol/L) did not enhance the neurotoxicity or lead to a further reduction of ATP levels.

Incubation of 10-day-old mixed cortex cultures from neonatal rats with 3-OH-glutaric acid significantly reduced the activity of complexes II and V of the respiratory chain, whereas incubation with glutaric acid reduced the activity of complex II only (Table 1). In this culture system, incubation with glutaric and 3-OH-glutaric acid did not reduce the intracellular ATP level, presumably because the cells were

Table 1 Mixed cortex culture. Activity^a of the respiratory chain

Complex ^b	Controls	Glutarate (4 mmol/L)	3-OH-Glutarate (4 mmol/L)	3-OH-Glutarate (2 mmol/L)
V	285 \pm 95	295 \pm 98	175 \pm 85*	222 \pm 105**
II + III	4 \pm 1	0	0	0

^a nmol/min/mg protein. The results give the mean value of 8 culture dishes each

^b The activity of complex I and IV was found to be normal in the presence of different metabolites

* $p < 0.01$; ** $p < 0.02$

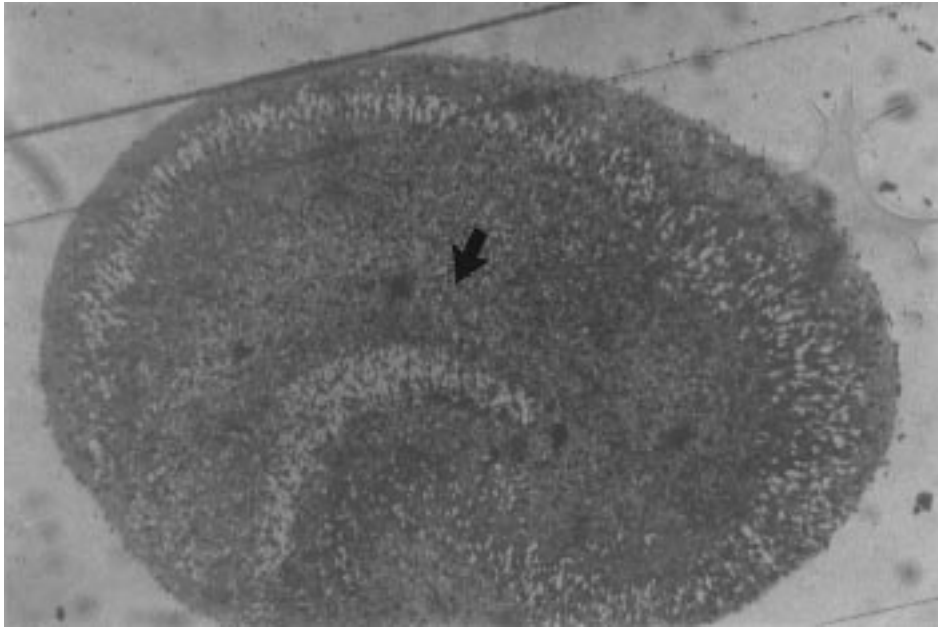


Figure 3 Hippocampal culture following incubation with 3-OH-glutaric acid (5 mmol/L), demonstrating vacuolation of neuron layers and vacuolar degeneration of glia cells (arrow). $\times 25$

not maximally stimulated (Table 2). Preliminary data indicate that 3-OH-glutaric acid specifically reduces the intracellular creatine phosphate levels (Table 2).

Administration of glutaric, 3-OH-glutaric and glutaconic acids at concentrations of 100 μ mol/L, 1 mmol/L or 3 mmol/L did not evoke membrane currents in frog oocytes expressing different glutamate receptors. Simultaneous administration of the metabolites with glutamate receptor agonists did not change agonist-induced membrane currents (Figure 4).

Table 2 Mixed cortex culture: ATP/CP concentrations^a

Compound ^b	Control	Glutarate (4 mmol/L)	3-OH-Glutarate (4 mmol/L)	3-OH-Glutarate (2 mmol/L)
ATP	11 \pm 5	10 \pm 4	7 \pm 3	9 \pm 2
CP	7 \pm 5	7 \pm 5	2 \pm 2*	3 \pm 3**

^a nmol/mg protein. The results give the mean value of 5 culture dishes each

^b ADP concentrations were found to be normal in the presence of different metabolites

* $p < 0.02$; ** $p < 0.05$

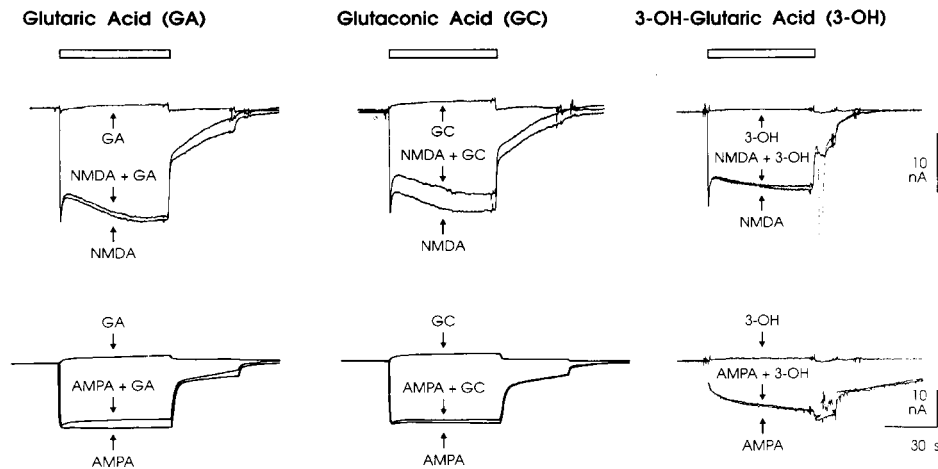


Figure 4 Effects of NMDA (100 μ mol/L plus 10 μ mol/L glycine), AMPA (100 μ mol/L), glutaric acid (GA, 1 mmol/L), 3-OH-glutaric acid (3-OH, 1 mmol/L) and glutaconic acid (GC, 1 mmol/L) on glutamate receptor currents in frog oocytes

In additional experiments using mixed cortex cultures (10 days) of postnatal rats, we showed (a) that only 3-OH-glutaric acid is enhancing the network activity; (b) that enhanced network activity can be prevented by NMDA receptor antagonists only; (c) that 3-OH-glutaric acid does not bind to NMDA receptors, (d) does not modulate glutamate binding to these receptors, (e) is not interacting with GABAergic receptors and (f) is presumably not leading to enhanced presynaptic glutamate concentrations, as can be taken from results obtained with autopsies (unpublished data).

DISCUSSION

Tissue slice cultures are the most physiological system to test glutamate toxicity, as they preserve the cytoarchitecture for more than 2 weeks with typical cell contact, transmitter interaction and function of glutamate carriers. Thus, as compared to cultivated hippocampal neurons, the glutamate toxicity is several times lower (Henneberry et al 1989).

Our results give evidence that 3-OH-glutaric acid is the relevant neurotoxin in glutaric aciduria type I. The metabolite induces neurodegeneration by activation of NMDA receptors, but does not directly interact with glutamate receptors as was shown electrophysiologically in different cell systems. In addition, it was found that 3-OH-glutaric acid does not modify the binding of glutamate to receptors, does not interact with GABA receptors and is presumably not producing enhanced presynaptic glutamate concentrations. The results do not exclude reduced presynaptic GABA

concentrations. Diminution of GABA-mediated inhibitory neurotransmission is thought to lead to neurodegeneration in the calf model of maple syrup urine disease (Dodd et al 1992).

Our results do not explain, how raised concentrations of 3-OH-glutaric acid produce an indirect activation of NMDA receptors. Reduced cellular ATP levels, interfering with the voltage-dependent Mg^{2+} -block of NMDA receptors, are presumably not the crucial mechanism as they were obtained in slice cultures by incubation with glutaconic or 3-OH-glutaric acid. Preliminary data indicate that 3-OH-glutaric acid specifically reduces the intracellular creatine phosphate levels. Kölker and colleagues (1998) demonstrated that the toxicity of 3-OH-glutaric acid is mediated by the NR2B subtype of NMDA receptors. These results may explain the regional distribution of neurodegeneration as found in patients (Monaghan and Cotman 1985; Wenzel et al 1997). Again this group did not find any direct ligand-receptor interaction. The regional distribution of cell death in hippocampal slices of different ages might be explained by an age-dependent expression of NMDA receptor subtypes.

Glutaric, glutaconic and 3-OH-glutaric acids additionally produce a vacuolar glial degeneration that in our hands could not be prevented by glutamate receptor antagonists. Excitotoxic mechanisms leading to degeneration of oligodendrocytes were recently described by McDonald and colleagues (1998).

It has been speculated that another glutamate receptor agonist with a high excitotoxic potential, quinolinic acid, may be responsible for neurodegeneration in glutaric aciduria type I (Heyes 1987), but quinolinic acid concentrations were found to be normal in the CSF of patients (Land et al 1992).

Our results allow us to speculate that mild energy deprivation is indirectly leading to activation of NMDA receptors. The functional properties, including susceptibility to glutamate or energy deprivation of different NMDA receptor subtypes, seem to be age dependent (Monyer et al 1994).

We believe that these results are the first steps in understanding the basic pathology of the disease and, if confirmed, will open a therapeutic window.

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